Array and method for analysing nucleic acid sequences.

The present invention relates to arrays for analysing nucleic acid sequences and to methods for analysing nucleic acid sequences using such an array.

In particular, the invention relates to arrays and methods for determining whether a specific nucleic acid sequence is present or absent in a nucleic acid sequence or mixture of nucleic acid sequences.

More in particular, the invention relates to an array and a method for determining the presence or absence, in genomic DNA or a sample of restriction fragments derived from genomic DNA, of sequences corresponding to unique restriction fragments that can serve as genetic markers, such as AFLP-markers.

The invention further relates to a method for preparing such an array, in particular in the form of a high density array for the detection of biological molecules, herein referred to as a "biochip".

A number of methods for analyzing nucleic acid sequences are known. In general, these methods comprise immobilization of the sequences to be analysed, for instance by blotting; hybridization of the sequences with a labeled DNA- or RNA-probe; stringency washes to remove non-hybridized material; followed by detection of those sequences that have hybridized with the probe.

Such techniques are often carried out after prior amplification -such as by PCR- of the starting nucleic acid sequences, usually a mixture of restriction fragments from a genomic DNA. The resulting mixture of amplified fragments is then separated, for instance on the basis of differences in length or molecular weight, such as by gel-electroforesis, and then visualised, i.e. by blotting followed by hybridization. The resulting pattern of bands is referred to as a DNA fingerprint.

Usually in DNA fingerprinting, fingerprints of closely related species, subspecies, varieties, cultivars, races or individuals are compared. Such related fingerprints can be identical or very similar, i.e. contain a large number of corresponding -and therefore less informative- bands.

Differences between two related fingerprints are referred to as "DNA polymorphisms". These are DNA fragments (i.e. bands) which are unique in or for a

fingerprint and/or for a subset of fingerprints. The presence or absence of such polymorphic bands, or the pattern thereof, can be used as a genetic marker, i.e. to identify a specific species, subspecies, variety, cultivar, race or individual, to establish the presence or absence of a specific inheritable trait, of a gene, or to determine the state of a disease.

For a further discussion and definitions of DNA-fingerprinting, DNA typing, DNA polymorphisms, genotyping, PCR and similar techniques, reference is made to the discussion of the prior art in EP-0 534 858 A1, incorporated herein by reference.

The abovementioned hybridization-based techniques require at least some prior knowledge of the sequence to be analysed, i.e. sufficient to provide a probe that can hybridize with the desired sequence(s). Such a probe must also be sufficiently selective to afford informative results. For instance, when analysing a plant genome, a probe that hybridizes with the "repeated" sequences within the genome will generally not provide any useful results, as such repeated sequences preclude typing unique polymorphisms.

A DNA-fingerprinting technique which requires no prior knowledge of the sequence to be analysed is described in the European patent application 0 534 858 by applicant, incorporated herein by reference. This technique, called selective restriction fragment amplification or AFLP, in general comprises the steps of:

(a) digesting a nucleic acid, in particular a DNA, with one or more specific restriction endonucleases, to fragment said DNA into a corresponding series of restriction fragments;

(b) ligating the restriction fragments thus obtained with at least one double-stranded synthetic oligonucleotide adapter, one end of which is compatible with one or both of the ends of the restriction fragments, to thereby produce tagged restriction fragments of the starting DNA;

(c) contacting said tagged restriction fragments under hybridizing conditions with at least one oligonucleotide primer;

(d) amplifying said tagged restriction fragment hybridized with said primers by PCR or a similar technique so as to cause further elongation of the hybridized primers along the restriction fragments of the starting DNA to which said

primers hybridized; and

(e) identifying or recovering the amplified or elongated DNA fragment thus obtained.

The thus amplified DNA-fragments can then be analysed and/or visualised, for instance by means of gel-electrophoresis, to provide a genetic fingerprint showing bands corresponding to those restriction fragments that have been linked to the adapter, recognized by the primer, and therefore amplified during the amplification step.

The AFLP-fingerprint thus obtained provides information on the specific restriction site pattern of the starting DNA. By comparing AFLP-fingerprints from related individuals, bands which are unique for each fingerprint can be identified. These polymorfisms are referred to as "AFLP-markers", and can again be used to identify a specific individual, cultivar, race, variety, subspecies or species, and/or to establish the presence or absence of a specific inherited trait, gene or disease state.

AFLP thus requires no prior knowledge of the DNA sequence to be analysed, nor prior identification of suitable probes and/or the construction of a gene library from the starting DNA.

For a further description of AFLP, its advantages, its embodiments, as well as the techniques, enzymes, adapters, primers and further compounds and tools used therein, reference is made to EP-A-0 534 858 and co-pending European applications 98.202.5496 and 98.202.4515, all by applicant and incorporated herein by reference. Also, in the description hereinbelow, the definitions given in paragraph 5.1 of EP-0 534 858 will be used, unless indicated otherwise.

Although AFLP is generally less time-consuming than hybridisation-based techniques, it still suffers from the disadvantage that the amplified fragments have to be separated (i.e. by gel-electrophoresis) and visualized (i.e. by generation of a fingerprint). These are very elaborate and time consuming procedures, which require special apparatus, such as electrophoresis and auto-radiography equipment. Thereafter, the fingerprints have to be analysed -nowadays generally performed by "reading" the fingerprint into a computer- to identify the polymorphic bands. Generally, this also requires to use of a known reference sample run at the same time in a parallel lane of the gel.

Because of these factors, AFLP can only be carried out in sufficiently equipped laboratories. Even so, it may take several days until results are obtained, even when routine tests following known protocols are carried out, such as on species or individuals of which the genome and/or relevant AFLP-markers are generally known.

A first aim of the invention is therefore to simplify these procedures, i.e. to provide a technique for analysing nucleic acid sequences which no longer requires the use of gel-electrophoresis and/or autoradiography.

This is achieved by providing a carrier-bound array of nucleic acid fragments, which can be used to analyse a sample of nucleic acid(s), such as a mixture of amplified restriction fragments of genomic DNA, by contacting the sample with the array under hybridizing conditions. This array-based detection can be used instead of gelectrophoresis/autoradiography, in particular for routine, high throughput genotyping.

The invention further provides a method for preparing such an array. In theory, this could be carried out by generating a sufficient number of conventional hybridization probes and binding them to a suitable carrier. This, however, is not practical for a number of reasons. For one, all these probes must be identified and prepared beforehand, essentially one at a time. This would make it very time consuming to prepare an array comprising a sufficiently large number of different probes, i.e. in the range of 1000-100.000 for the micro-array's disclosed herein.

Also, these probes would have to be selective. If, for instance, all restriction fragments from a starting genomic DNA were to be used as probes on an array, large parts of the array would not be informative, as the sequences bound thereto would be too abundant in the nucleic acid sequence(s) to be analysed. Also, the sheer number of fragments obtained by restricting a genomic DNA would make it too time-consuming to prepare or analyse (i.e. "read") such an array.

The invention also solves this problem, in that it allows -during the preparation of nucleic acid sequences for use in the array- to select only, or essentially only, those sequences that correspond to fragments/bands of interest, i.e. to select genetic markers. The invention also allows - simultaneously - the identification and preparation of a large number of such informative fragments, and to selectively prepare and purify these fragments in amounts sufficient for binding to the carrier.

According to the invention, this is carried out by analysing the genomic DNA of two or more related individuals using AFLP, identifying polymorphisms ("AFLP-markers") within the genome, amplifying and isolating the nucleic acid sequences corresponding to these AFLP-markers, and binding the amplified sequences to specific areas of a carrier, thus providing an array comprising essentially only nucleic acid sequences that correspond to AFLP-markers.

This array can then be used to analyse a sample of nucleic acid(s) -such as a genomic DNA or restriction fragments thereof- derived from the same or a genetically related individual, by contacting the sample with the array under hybridizing conditions. The nucleic acid sequence(s) to be analysed will then (only) hybridize with those parts of the array that carry an essentially homologous sequence, i.e. the same AFLP-marker, or at least a sequence with a high degree of homology with the marker. Thus, by analysing to which parts of the array (i.e. to which AFLP-markers) the nucleic acid sequence(s) to be analysed has or have hybridized, the presence of absence of said marker in the sample can be established.

In other words, the invention makes it possible to test a sample of nucleic acid(s) directly for the presence of a large number of polymorphic fragments or bands - i.e. as many as are bound to the carrier- without the need of generating and analysing a DNA-fingerprint.

The invention also makes it possible to test simultaneously for a large number of "unrelated" markers (i.e. markers which can normally not be detected in a single AFLP-reaction or fingerprint) by incorporating these different markers into a single array.

Other objects and advantages of the invention will become clear from the description hereinbelow.

H. Himmelbauer et al., Mammalian Genome 9, 611-616 (1998) describe a method for the identification and mapping of polymorphic markers, using "*a modification of the AFLP technique*" called the "*IRS-PCR system*". According to this method, genomic (mouse) DNA is restricted using a single restriction enzyme (*Sac*I or *Bam*HI), amplified in a PCR using adapters and primers, after which the amplicons thus obtained are hybridized with a gridded genomic library (BAC-clones) to identify

strain-specific differences. Positive clones can then be used to generate genotyping information, i.e. by hybridizing fragment mixtures derived from individuals of a backcross population against the positive clones, or by amplifying individual clones for hybridization against the complex fragment mixtures derived from individuals pf a backcross population.

In the invention, compared to the method of Himmelbauer, the markers are generated by restricting with two restriction enzymes, i.e. a rare and a frequent cutter. Also, the invention does not require the preparation of a BAC-library, nor of a subsequent hybridization against a backcross.

Also, Himmelbauer et al. do not suggest to use the IRS-PCR- derived clones in an array. The array used by Himmelbauer, a high density spotted filter grid of genomic BAC-clones, is prepared using conventional complex probe hybridization. Also, this array is not (and cannot be) used to scan a DNA sample directly for the presence of markers. Instead, this grid is used in the identification of markers (i.e. by further hybridisation with a backcross), which markers are then used for genome mapping.

The art also describes oligonucleotide arrays, vide for instance WO 97/27317, WO 97/22720, WO 97/43450, EP 0 799 897, EP 0 785 280, WO 97/31256, WO 97/27317 and WO 98/08083.

Such arrays, which include the Genechips™ array, the Affymetrix DNA chip and the VLSIPS™ array, can have nucleotide densities of more than 100-10.000 per cm^2 or more and are generally prepared by "building up" the oligonucleotides on the solid support using sequential solid phase nucleic acid synthesis techniques. However, as this is difficult and time-consuming, even when using automated equipment, there is a practical limit to the size of the oligonucleotides on the array, i.e. of about 100 nucleotides, usually about 10-50 nucleotides, usually without variation in size. The use of such small oligonucleotides can lead to a relatively large occurrence of mismatch events, which reduces selectivity and increases the background noise.

Because of this, these known arrays generally require several of the attached oligonucleotides to detect a target sequence. Also, they do not directly provide data on the presence of specific markers, but require substantive analysis of the signal pattern,

usually by comparison to known results or a reference using sophisticated computer algorithms.

In a first aspect, the present invention relates to an array for analysing a nucleic acid sequence or a mixture of nucleic acid sequences, comprising:

a) a carrier; and

b) at least two different nucleic acid sequences bound to said carrier, in which each of the nucleic acid sequences bound to the carrier comprises at least a nucleic acid sequence that corresponds to the sequence of a restriction fragment obtainable by restricting a genomic DNA with at least one frequent cutter restriction enzyme and at least one rare cutter restriction enzyme.

More particular, the invention relates to such an array in which at least 50%, preferably at least 70%, more preferably at least 90% of the nucleic acid sequences bound to the carrier comprise the sequence of a restriction fragment that corresponds to an AFLP-marker.

In a further aspect the invention relates to a method for providing an array of nucleic acid sequences bound to a carrier, comprising the steps of:

a) identifying an AFLP-marker;

b) providing a nucleic acid sequence that comprises a restriction fragment sequence corresponding to said AFLP-marker;

c) attaching the nucleic acid sequence to the carrier; and

d) repeating steps a) to c) for different AFLP markers to build up an array.

More particularly, the invention relates to such a method comprising the steps of:

a) identifying a polymorphic band in an AFLP-fingerprint;

b) isolating a nucleic acid sequence from said polymorphic band;

c) optionally further amplifying, purifying and/or modifying the nucleic acid sequence; and

d) attaching the nucleic acid sequence to the carrier.

e) repeating steps a) to d) for different polymorphic bands to build up an array.

In a yet another aspcect, the starting DNA used to generate the restriction fragments that are bound to the carrier are not derived from genomic DNA, but from at

least one cDNA. Generally, an array according to this aspect of the invention comprises:

a) a carrier; and

b) at least two different nucleic acid sequences bound to said carrier, in which each of the nucleic acid sequences bound to the carrier comprises at least a nucleic acid sequence that corresponds to the sequence of a restriction fragment obtainable by restricting at least one cDNA with at least one frequent cutter restriction enzyme and at least one rare cutter restriction enzyme.

A method of the invention for preparing such a cDNA-based array generally comprises the steps of:

a) providing a nucleic acid sequence that comprises at least one restriction fragment that has been derived from at least one cDNA.

b) attaching the nucleic acid sequence to the carrier; and

c) repeating steps a) and b) for different cDNA-derived restriction fragments to build up an array.

More particularly, the invention relates to such a method comprising the steps of:

a) analysing at least one cDNA using AFLP-methodology to provide a cDNA-AFLP-fingerprint, said fingerprint comprising at least one, and usually a plurality, of bands;

b) isolating from at least one of said bands at least one nucleic acid sequence;

c) optionally further amplifying, purifying and/or modifying the nucleic acid sequence; and

d) attaching the nucleic acid sequence to the carrier.

e) repeating steps a) to d) for different bands and/or for different cDNAs to build up an array.

In yet another aspect, the invention relates to method for analysing a nucleic acid (sequence) or a mixture of nucleic acids (nucleic acid sequences), comprising contacting said nucleic acid or mixture with an array as described herein.

Other aspects and embodiments of the invention will become clear from the description and experimental part hereinbelow.

In the description, the nucleic acid sequences bound to the carrier will be indicated as "*Array-bound Nucleic Acid Sequence(s)*" or "ÄNAS", and the restriction fragments present therein will be indicated as "*Restriction Fragment Sequence(s)*" or "RFS". Usually, each Array-bound Nucleic Acid Sequences will comprise (only) one Restriction Fragment Sequence, and optionally further nucleic acid sequences or structural elements as described below, bound to the Restriction Fragment Sequence. When Array-bound Nucleic Acid Sequences are referred to hereinbelow as "different", it means that these Array-bound Nucleic Acid Sequences contain different Restriction Fragment Sequences.

The array preferably comprises at least 10, more specifically at least 100, more preferably at least 1000 different Array-bound Nucleic Acid Sequences. For a "high-density array" or "micro-array", the total number of Array-bound Nucleic Acid Sequences will be in the region of 100 - 100.000.

These Array-bound Nucleic Acid Sequences will generally be bound to the carrier in such a way that each Array-bound Nucleic Acid Sequence is attached to, and corresponds with, a specific, distinct part of the carrier, so as to form an independently detectable area on the carrier, such as a spot or band. This makes it possible to "read" the array by scanning (i.e. visually or otherwise) the areas to which the Array-bound Nucleic Acid Sequence (i.e. the marker) of interest is attached.

Preferably, the Array-bound Nucleic Acid Sequences are bound to the carrier in accordance with a predetermined, regularly distributed pattern, in which for instance related Array-bound Nucleic Acid Sequence (i.e. related markers) can be grouped together, i.e. in one or more lines, columns, rows, squares, rectangles, etc, preferably in an "adressable" form. This further facilitates analysis of the array.

The density of the different Array-bound Nucleic Acid Sequences will generally be in the region of 1-100,000 different markers/cm^2, usually 5-50,000 markers/cm^2, generally between 10-10,000 markers/cm^2.

In general, each of the Array-bound Nucleic Acid Sequences on the array will correspond to a specific polymorphic band or marker, i.e. as derived from an AFLP-fingerprint of genomic DNA of a specific individual. Usually, the array will comprise sets of one or more of such markers taken from a single fingerprint, or at least taken

from fingerprints of a single individual.

Often, the array will be build up of one or more of such individual sets, each taken from an AFLP-fingerprint of a different but related individual. By "related individuals" is meant herein that these individuals are such that useful or desired information can be obtained by comparing their DNA-fingerprints, more specifically their AFLP-fingerprints. Usually, this means that these individuals share or have related inherited properties or traits (including genetic markers) and/or have nucleic acid sequences in their genome (such as genes) which are the same or related. In practice, related individuals will usually stem from the same family, genus, species, subspecies, variety, cultivar or race, depending upon the purpose of the comparison.

In the array's of the invention, the markers taken from one individual, and the sets of markers taken from related individuals, will usually be arranged on the array in a predetermined, regular pattern.

Usually, the markers will be derived from a limited number of related individuals, which have been selected in such a way that they represent the genetic diversity within the group of interest (i.e. family, genus, species, subspecies, cultivar, race or variety) in the best possible way. This selected set of individuals is called the "genotyping collection".

Preferably the array will contain a majority or even all the markers from a genotyping collection that are characteristic for the presence or absence of the one or more traits or properties of interest. For instance, an array may contain all or most markers characteristic for the dominant, the recessive and any or all allelic forms of one or more genes or traits of interest, as may be present within different individuals from the same family, genus or species.

An array of the invention can (also) contain sets of markers that correspond to different (i.e. genetically unrelated) traits or properties, and such an array can be used to analyse an individual (genome) for the presence of absence of all these properties simultaneously. However, such unrelated markers will usually still have been obtained from within one genotyping collection, i.e. from individuals belonging to the same family, genus or preferably species, i.e. so as to provide -for instance- a "maize-array", a "tomato-array", a "wheat-array" etc..

In one embodiment, the AFLP-markers present on the array have been taken from or will be representative of different subspecies, varieties, cultivars, lines or races of the same species.

An array of the invention can also contain markers representative of a certain genetic state of an individual, such as the presence or absence of a disease state, i.e. of oncogenes and of genetically determined diseases.

As already mentioned above, besides arrays based on restriction fragments derived from genomic DNA - e.g. based on polymorphic fragments/genetic markers - the invention also provides arrays based on (restriction fragments derived from) cDNA.

According to this aspect of the invention, the RFS present in the ANAS will be a restriction fragment obtained by restricting at least one cDNA with at least one restriction enzyme, and preferably with at least one frequent cutter restriction enzyme and at least one rare cutter restriction enzyme as described herein.

Usually, prior to attachment to the array, the cDNA-derived restriction fragments thus obtained are amplified, preferably using AFLP. Such AFLP-amplification of cDNA is generally referred to as "cDNA-AFLP" and can be carried out essentially as described above for the AFLP-amplification of genomic DNA and/or by using any cDNA-AFLP protocol known per se, to provide a cDNA-derived AFLP-fingerprint.

One or more of the bands from this cDNA-AFLP fingerprint may then be isolated from the gel and bound to the array, e.g. after re-amplification and/or incorporation into an ANAS, essentially as described for the genomic DNA.

This may be carried out for different bands obtained from the same cDNA, and/or for bands from one or more different cDNAs. Also, the one or cDNAs used to provide the RFS may be obtained from (mRNAs derived from) one individual (e.g. from different cells, parts, tissues or organs) and/or from two or more individuals, e.g. individuals belonging to same race, variety, species, genus, family etc.., with the same or different phenotypical characteristics. Also, the cDNAs may be obtained from (mRNA derived from) healthy individuals and/or from diseased individuals; and/or from individuals at different stages of development.

Furthermore, although the genomic DNA based arrays and the cDNA based

arrays are discussed separately hereinabove, it will be clear to the skilled person that an array of the invention may also contain both one or more restriction fragments derived from genomic DNA as well as one or more restriction fragments derived from cDNA.

Although preferably, each Array-bound Nucleic Acid Sequence on the array will correspond to a polymorphic band of interest (i.e. a marker) or an informative cDNA-derived band, the presence on the array of some non or less informative Array-bound Nucleic Acid Sequences (for instance corresponding to non-polymorphic bands or to markers that are too abundant to provide useful information) is not excluded. However, these will preferably constitute less than 50%, preferably less than 30%, more preferably less than 10% of all Array-bound Nucleic Acid Sequences present on the array. It is also included that some or most of the Array-bound Nucleic Acid Sequences may be informative for one specific application or genome, but not for another. However, preferably 95-100% of all Array-bound Nucleic Acid Sequences will correspond to or contain an AFLP-marker.

The manner in which the Array-bound Nucleic Acid Sequences and the Restriction Fragment Sequences are obtained is further described in the Experimental Part below.

In general, the Restriction Fragment Sequences are characterized in that they are obtainable/obtained by cutting a starting DNA, usually a genomic DNA or cDNA, with at least one "frequent cutter" restriction enzyme and at least one "rare cutter" restriction enzyme. These fragments are then bound to adapters and amplified using (usually selective) primers. The thus amplified fragments are visualized in a DNA-fingerprint, and polymorphic bands are identified, i.e. by comparison with the fingerprint(s) of one or more related individuals or to a database. The restriction fragments present in these polymorphic bands are then individually isolated (by cutting them out from the gel) and optionally further purified and/or amplified, after which they are attached to a specific, distinct area of the carrier, optionally after modification of carrier surface and/or of the fragment to allow or promote such attachment.

Therefore, generally speaking, the invention uses AFLP-methodology both to select and to prepare (i.e. to amplify and to isolate) the nucleic acid sequences to be attached to the array, and to do so simultaneously. The use of AFLP in the invention

also makes it possible to identify and prepare, at the same time, markers from related individuals (i.e. from one genotyping collection) i.e. by running parallel AFLP-reactions and visualizing these reactions in adjacent lanes of the same gel. In this way, a micro-array containing a large number of markers and/or containing all relevant markers from a genotyping collection can be build up very efficiently.

As in AFLP, two different restriction enzymes are used to digest the starting (genomic) DNA, i.e. the "frequent cutter", which serves the purpose of reducing the size of the restriction fragments to a range of sizes which are amplified efficiently, and the "rare cutter" which serves the purpose of targeting rare sequences. For both, reference is made to EP-A-0 534 858 and EP-A-0 721 987 by applicant, incorporated herein by reference.

Examples of suitable frequent cutter enzymes are *Mse*I and *Taq*I. Examples of commercially available rare cutters are *Pst*I, *Hpa*II, *Msp*I, *Cla*I, *Hha*I, *Eco*RII, *Bst*BI, *Hin*P1, *Mae*II, *Bbv*I, *Pvu*II, *Xma*I, *Sma*I, *Nci*I, *Ava*I, *Hae*II, *Sal*I, *Xho*I and *Pvu*II, of which *Pst*I, *Hpa*II, *Msp*I, *Cla*I, *Eco*RII, *Bst*BI, *Hin*P1 and *Mae*II are preferred.

The AFLP-reaction will usually be carried out following known protocols, for which reference is made to EP-A-0 534 858, incorporated herein by reference.

The Restriction Fragment Sequence (with the AFLP-adapters) will generally have a size that can be detected as an individual band in an AFLP-fingerprint, i.e. in the range of 50 – 1200 base pairs. It will be clear that, as the Restriction Fragment Sequence are separated by gel-electrophoresis, they will be of different sizes.

Also, it may be possible to use as the Restriction Fragment Sequence only a part of a restriction fragment obtained as/from a band in the AFLP fingerprint. Such a partial sequence may for instance be obtained by (further) restricting the restriction fragment(s) isolated from the AFLP gel with one or more restriction enzymes, i.e. usually with other restriction enzymes than the one or two orginally used to generate the restriction fragments from the starting genomic or cDNA, including but not limited to synthesized oligonucleotides based and/or derived thereof. For this purpose, any desired and/or pre-determined restriction enzyme or enzyme combination may be used; suitable restriction enzymes include, but are not limited to, the frequent cutters and rare cutters mentioned above, IIS-type restriction enzymes.

In general, such a partial sequence generated by (further) restricting the restriction fragments obtained from the AFLP gel may have any suitable size, up to the size of the original restriction fragment (i.e. when no recognition site for the restriction enzyme used is present in the restriction fragment). Usually, however, these partial sequences will be smaller than the restriction fragments, i.e. the range of 10 – 100 base pairs.

The use of such smaller, but still specific, partial sequences may have some advantages, such as avoiding cross-hybridization between sequences showing homologous regions. Also, for the purposes of the description herein, such partial sequences should be considered as encompassed within the term "Restriction Fragment Sequence" as used herein.

Preferably, the Array-bound Nucleic Acid Sequence comprises single-stranded DNA, although the use of double-stranded DNA as Array-bound Nucleic Acid Sequences is also within the scope of the invention.

The Array-bound Nucleic Acid Sequence will at least comprise one (and usually only one) Restriction Fragment Sequence, and can further contain other sequences or structural elements, often at the end(s) of the Restriction Fragment Sequence-sequence. These include AFLP-adapter sequences (one or two) and/or other nucleic acid sequences, as well as groups or functionalities that can be used for attaching the Array-bound Nucleic Acid Sequence to the array (hereinbelow referred to as "binding elements").

The adapter sequences will usually be present at the end(s) of the Array-bound Nucleic Acid Sequence, and may be the adapters used in the AFLP-reaction with which the original genomic DNA as amplified, and/or used to amplify the sample to be analysed. However, they preferably contain (adapter) sequences different thereto, for the reasons given in the Experimental Part below.

The adapters may also have been modified to contain groups or functionalities that can be used for attaching the Array-bound Nucleic Acid Sequence to the array, so as to make the adapter into a binding element.

The binding elements may be present at the end(s) of the Restriction Fragment Sequences (i.e. replacing the adapters) but may also be present in or on the Restriction

Fragment Sequence itself, depending upon the technique used for binding the Array-bound Nucleic Acid Sequence to the array, as further described below.

The carrier for the array may be any solid material to which nucleic acid sequences can be attached, including porous, fibrous, woven and non-woven materials, as well as composite materials. Also, semi-solid materials such as gels or matrices (for instance as used in chromatography) may be used, although this is not preferred.

Suitable carriers include, but are not limited to, those made of plastics, resins, polysaccharides, silica or silica-based materials, functionalized glass, modified silicon, carbon, metals, inorganic glasses, membranes, nylon, natural fibers such as silk, wool and cotton, and polymer materials such as polystyrene, polyethylene glycol tetraphthalate, polyvinyl acetate, polyvinyl chloride, polyvinyl pyrrolidone, polyacrylonitrile, polymethyl methacrylate, polytetrafluoroethylene, butyl rubber, styrenebutadiene rubber, natural rubber, polyethylene, polypropylene, (poly)tetrafluoroethylene, (poly)vinylidenefluoride, polycarbonate and polymethylpentene. Further suitable support materials are mentioned for instance mentioned in US-A-5,427,779, WO 97/22720, WO 97/43450, WO 97/31256, WO 97/27317 and EP 0 799 897.

Preferred carrier materials are glass and silicon.

Preferably, the carrier will have an essentially flat, rectangular shape, with the Array-bound Nucleic Acid Sequences bound to one surface thereof. However, any other suitable two- or three-dimensional form may also be used, such as a disc, a sphere or beads, or materials or structures that allow a liquid medium containing the sample to be analysed to pass or flow through the carrier, such as columns, tubes or capillairies, as well as (macro)porous-, web- or membrane-type structures, including the flow-through genosensor devices referred to in WO 97/22720.

The size of the array, as well as of the individual areas corresponding to each of the different Array-bound Nucleic Acid Sequences, may vary, depending upon the total amount of Array-bound Nucleic Acid Sequence, as well as the intended method for analysing the array.

For an array that is to be inspected visually, the total array and the separate areas thereon will be of such a size that they can be seen and distinguished with the naked eye or through a microscope, i.e. in the range of 1 to 500 cm^2 for the total array,

and 0.01 to 0,1 cm^2 for the individual areas.

Arrays that are analysed using other types of (usually automated) scanning equipment may be of smaller size, and are preferably in the form of high-density or micro-arrays, i.e. in the range of 1 - 10 cm^2 for the total array, 0.001 – 0.1 cm^2 for the individual areas. This allows hybridization to be carried out in a small volume on a small sample, or even the use of flow-through techniques.

The Array-bound Nucleic Acid Sequences may be bound to the carrier in any manner known per se, and the specific technique used will mainly depend upon the carrier used. Binding may be at the 3'-end, at the 5'-end, or somewhere else on the Restriction Fragment Sequence/Array-bound Nucleic Acid Sequence, as appropriate.

Preferably, the Array-bound Nucleic Acid Sequence will be covalently bonded to the array, i.e. by a suitable chemical technique. As mentioned above, for this purpose, the Array-bound Nucleic Acid Sequence and/or the carrier may be modified to carry one or more binding groups or elements. For instance, the surface of the carrier may be activated to carry one or more groups such as carboxy, amino, hydroxy, etc..

Suitable methods for attaching the Array-bound Nucleic Acid Sequences to the carrier will be clear to the skilled person. In general, any method for attaching a nucleic acid to a solid support can be used, including the methods described in US-A-5,427,779; US-A-4,973,493; US-A-4,979,959; US-A-5,002,582; US-A-5,217,492; US-A-5,525,041; US-A-5,263,992; WO 97/46313 and WO 97/22720, as well as the references cited therein.

As an example of covalent attachment, coupling can proceed using photoreactive groups such as N-oxy-succinimide, in which either the Array-bound Nucleic Acid Sequence is derivatized with a photoreactive group and attached to the surface, or the surface is first treated with a photoreactive group, followed by application of the Array-bound Nucleic Acid Sequence, for instance in N-terminal amino-modified form. A suitable protocol, following the general method described in Amos et al., Surface Modification of Polymers by Photochemical Immobilization, The 17th Annual Meeting of the Society of Biomaterials, May 1991, Scottsdale AZ, given in WO 97/46313, incorporated herein by reference.

Other covalent binding techniques involve the use of 3'-aminopropanol-groups

or epoxysilane-amine chemistry, for instance as described in WO 97/22720, also incorporated herein by reference.

An example of a strong, but non-covalent binding technique involves the attachment of a biotinylated Array-bound Nucleic Acid Sequence onto a carrier coated with streptavidin.

In order to create small, distinct, adressable areas of each of the Array-bound Nucleic Acid Sequence on the array, masking techniques or known microdispensing techniques may be used, for instance as described in WO 97/46313 and WO 97/22720.

After attachment of the Array-bound Nucleic Acid Sequences to the carrier, the array will generally be ready for use.

In a further aspect, the invention relates to a method for analysing a nucleic acid sample using the array of the invention. In general, this method comprises contacting the sample to be analysed with the array under hybridizing conditions, so that the one or more of the nucleic acid sequence(s) present in the sample may bind to the one or more of the Array-bound Nucleic Acid Sequences on the array, more specifically with the Restriction Fragment Sequences present in the Array-bound Nucleic Acid Sequence. This method is described in more detail in the Experimental Part below.

Usually, a nucleic acid sequence or mixture will be analysed that is suspected to comprise at least one sequence or fragment that corresponds to a Restriction Fragment Sequence (i.e. an AFLP-marker) present on the array used. In this context, "corresponds" means a sequence homology of at least 70%, more preferably at least 85%, specifically 95%-100%.

In general, the method of the invention is based on the hybridisation of sequences in the sample to be analysed with the Restriction Fragment Sequence. In other words, in the invention, the target sample is probed directly with the pre-selected sequences/markers of interest, so that a positive hybridization event or signal is directly indicative of the presence of said marker in the target sample. Also, as these markers are unique sequences with low abundance in the target genome, generally a high selectivity can be obtained,

Also, in a highly preferred embodiment of the invention, in analysing a target

genome, said genomic DNA is subjected to "AFLP" prior to hybridisation to the array, in which by "ÄFLP" in this context is more generally meant that the starting DNA is cut using at least one restriction enzyme and then amplified using adapters and primers, of which at least one contains at least one selective base at the 3'-end. This leads to a further reduction of sample complexity, giving less background noise.

Even more preferably, in the AFLP amplification prior to hybridization, the same frequent cutter and rare cutter are used as were used in generating (at least some of) the Restriction Fragment Sequence, and most preferably a similar protocol is followed, using the same (selective) primers. In this way, the amplified sample will contain, and essentially only contain, fragments that exactly correspond to the Restriction Fragment Sequence on the array (i.e. besides further non-polymorphic fragments that are not expected to hybridize with (the RFS on) the array). This improves specificity and reliability even further.

Suitable hybridisation conditions (i.e. buffers used, salt strength, temperature, duration) can be selected by the skilled person, on the basis of experience or optionally after some preliminary experiments. These conditions may vary, depending on factors such the Array-bound Nucleic Acid Sequences present on the array (size of the Restriction Fragment Sequence, CG-content etc.), and the sample to be analysed.

Suitable hybridisation conditions are for instance described in Sambrook et al., *Molecular Cloning: A Laboratory manual*, (1989) 2nd. Ed. Cold Spring Harbour, N.Y.; Berger and Kimmel, "Guide to Molecular Cloning Techniques", *Methods in Enzymology*", (1987), Volume 152, Academic Press Inc., San Diego, CA; Young and Davis (1983) *Proc. Natl. Acad. Sci.(USA)* 80: 1194; *Laboratory Techniques in Biochemistry and Molecular Biology, Vol.24, Hybridization with Nucleic Acid Probes*, P. Thijssen, ed., Elsevier, N.Y. (1993), as well as WO 97/43450. EP-A-0 799 897, WO 97/27317, WO 92/10092, WO 95/1195, WO 97/22720 and US-A-5,424,186, all incorporated herein by reference.

Suitable hybridisation conditions comprise temperatures between 25-70°C, preferably 35-65°C, a duration of between 1 minute and 30 hours, preferably about 30 minutes to 2 hours, and using known hybridization buffers, such as salt-, Tris- or citrate- contaning buffers, etc., and may for example vary from 6X SSPE-T at about

40°C to 1X SSPE-T at 37°C down to as low as 0.25X SSPE-T at 37-50°C.

The hybridisation conditions are preferably chosen such that only those nucleic acid sequences in the target sample that have more than 70%, preferably more than 80%, more preferably more than 90% homology, and in particular 95-100% homology with the Restriction Fragment Sequences, will hybridize with the Array-bound Nucleic Acid Sequence. These will generally be "moderate" or preferably "stringent" hybridisation conditions. Such stringent conditions can be as described in EP 0 799 897.

After hybridization, the array is washed to remove unwanted compounds, in particular any nucleic acid sequences not hybridized with the Array-bound Nucleic Acid Sequences on the array. Thereafter, the array is analysed to determine to which areas on the array (i.e. to which Array-bound Nucleic Acid Sequences/Restriction Fragment Sequences) the nucleic acid sequence(s) from the sample has/have hybridized. These area's will generally be detected as a positive signal indicating the presence of the marker in the sample.

The analysis of the array may be carried out in any manner known per se, including optical techniques, spectroscopy, chemical techniques, biochemical techniques, fotochemical techniques, electrical techniques, light scattering techniques, colorimetric techniques, radiography techniques, etc., as long as they can indicate the presence of a hybridization event. Suitable techniques are for instance described in WO 97/27317, WO 97/22720, WO 97/43450, EP 0 799 897, WO 97/31256, WO 97/27317 and WO 98/08083.

Usually, a technique using detectable labels will be used. Such a label will generally be attached to the nucleic acid sequence(s) to be analysed, so that -after hybridization with the array- those areas of the array which show the presence of the label correspond to a positive hybridization event.

Suitable labels are for instance described in WO 97/27317, WO 97/22720, WO 97/43450, EP 0 799 897, WO 97/31256, WO 97/27317 and WO 98/08083 and include fluorescent labels, phosphorescent labels, chemoluminescent labels, bioluminescent labels, chemical labels, biochemical labels such as enzymes, biological labels such as biotin/streptavidin, radioisotopes, spin or resonance labels, metal colloids

such as gold, magnetic beads, chromogens, dyes, and similar labels.

These labels may be incorporated into the target nucleic acids during amplification, for instance by using labelled primers or nucleotides. Also, primers or nucleotides carrying binding groups to which a label subsequently may be attached can be used in the amplification reaction.

Alternatively, the target nucleic acids may be end-labelled after amplification, for instance as described in WO 97/27317. Furthermore, so-called "indirect" labels may be used, which are joined to the target sequence/Array-bound Nucleic Acid Sequence-duplex after hybridisation, again as for instance described in WO 97/27317.

Detection and optionally recording of positive signals on the array is carried out in a manner known per se, usually depending on whether a label is used, and if so, the type thereof. For instance, the array may be inspected visually or by (confocal) microscopy; by spectroscopy; using photographic film, electronic detectors or a CCD camera; by colorimetric or (bio)chemical assay; or by any other suitable method, for which again reference is made to WO 97/27317, WO 97/22720, WO 97/43450, EP 0 799 897, WO 97/31256, WO 97/27317 and WO 98/08083. Automated scanning equipment based upon such techniques may also be used.

Optionally, the relative intensity or absolute magnitude of a positive hybridisation signal for a binding site on the array may be used as a relative indication or an absolute measure of the amount of the corresponding fragment present in the original sample, for instance as described in WO 98/08083.

The analysis of the hybridization (pattern) to the array may as such provide useful results, i.e. show the presence or absence of a genetic marker or genetic trait of interest, identify an individual, or otherwise provide information on the individual analysed, such as to which strain, variety, cultivar or race it belongs. It may also directly indicate the presence or absence of a disease state.

Optionally, the data obtained from "reading" the array may also be processed further, i.e. by comparing it to references, to earlier results or to a database, optionally using computer algorithms.

Advantageously, the array of the invention can be used to replace conventional fingerprinting/autoradiography analysis in AFLP. This aspect of the invention

comprises steps (a) - (e) of the general AFLP-method described above, in which step (e) is carried out by contacting the (mixture of) amplified or elongated DNA fragment(s) obtained in step (d) with an array as described herein.

Compared to conventional fingerprinting/autoradiography, the use of an array generally will be faster than using fingerprinting/autoradiography, and several markers that would require generating several separate fingerprints could be combined into a single array. This makes the arrays of the invention especially suited for routine and/or high throughput screening, for instance in plant breeding.

Also, the array of the invention can conveniently be provided as a kit of parts comprising the array and other components for use with the array, such as restriction enzymes, polymerase(s), adapters, primers, buffers, nucleotides, labels or other detection agents, containers/packaging and manuals. The array of the invention may even be in the form of a hand-held device such as a dipstick.

The array of the invention may be re-usable, usually through regeneration to remove the hybridized sequences. A kit of the invention may therefore also contain agents that can be used for such regeneration.

The array of the invention can be used to analyse any kind of nucleic acid sequence or mixture of nucleic acid sequences, including, but not limited to, plant-derived sequences, animal-derived sequences, human-derived sequences, microbial sequences, yeast sequences, sequences from fungi and algi, and viral sequences, depending upon the origin of the restriction fragment sequences bound to the array, including but not limited to whether the restriction fragments bound to the array are derived from genomic DNA or cDNA (or both).

Also, the array may be used to analyse DNA sequences, including genomic DNA, cDNA, structural genes, regulatory sequences and/or parts thereof; as well as RNA, including mRNA, optionally by analogous modification of the method given above.

The nucleic acid sample analysed with the array may be a sample as isolated directly from a living or dead organism or from tissue or cells. Preferably however, prior to hybridisation with the array, the nucleic acid sample is restricted with one or more restriction enzymes, preferably the same two restriction enzymes used in

generating the array, although this is not mandatory.

Also, the nucleic acid sample (or parts of it) may be amplified prior to hybridisation with the array, using any type of suitable amplification technique, preferably a PCR-based technique. As already mentioned above, conveniently AFLP may be used, preferably using the same adapters and primers as used in generating the array. This not only allows the use of known, reliable protocols, but can also reduce sample complexity, thereby improving the signal-to-noise ratio.

However, it should be understood that the array of the invention generally comprises a set of specific probes/markers bound to a carrier, so that the array can be used to probe any nucleic acid sample for the presence of a corresponding sequence. This is independant of the form which the nucleic acid sample may take (i.e. full genomic DNA, cDNA, or fragments thereof), or whether it has been amplified, and if so, by which amplification technique.

It should further be understood that, compared to fingerprinting, the use of the array no longer is, or has to be, based on detecting differences in fragment length, as sequences of interest can be detected directly. Therefore, the arrays of the invention, once prepared, are more broadly applicable than in AFLP only.

Also, although the array of the invention is primarily intended for detection and analysis, it may also be used to quantitatively prepare or isolate DNA, RNA or any fragment thereof, i.e. by releasing the hybridized sequences from the array after removal of unwanted sequences. The sequences thus obtained may then be used or analysed further, for instance to determine their sequence.

In principle, arrays of the invention can be developed for, and can be used for, any purpose for which a polymorphic marker can be used and/or identified. This includes, but is not limited to, all the uses described in the art for polymorphic markers in known DNA-fingerprinting, genotyping, profiling and DNA-identification techniques. The arrays of the invention are of course especially suited in those applications for which an AFLP-marker can be used and/or identified, including those mentioned above and in EP-A-0 534 858 and the co-pending European applications 98.202.5496 and 98.202.4515.

Also, besides the applications already mentioned, a cDNA-based array of the

invention can be used for any purpose for which the use of cDNA-AFLP is envisaged, including but not limited to applications such as expression profiling, functional genomics, and gene mapping. For any of these applications, it is envisaged that - as with cDNA AFLP - a cDNA-based array may be used to determine both qualitatively as well as quantitatively - e.g. based on the strength of the hybridisation signal obtained with the array - the presence of one or more specific nucleotide sequences in a starting sample. These may include both DNA-sequences as well as RNA-sequences, including expresssion-dependant RNA sequences such as mRNAs.

Possible fields of use of both the genomic DNA-based as well as the cDNA-based arrays are for instance plant and animal breeding, variety or cultivar identification, diagnostic medicine, disease diagnosis in plants and animals, identification of genetically inherited diseases in humans, family relationship analysis, forensic science, organ-transplant, microbial and viral typing such as multiplex testing for strains of infectious diseases; as well as the study of genetic inheritance, gene expression, mutations, oncogenes and/or drug resistance; or for mRNA detection.

Arrays of the invention may further be developed for and used in any other application for which known nucleotide arrays are used or envisaged. These include the applications mentioned in for example WO 97/27317, WO 97/22720, WO 97/43450, EP 0 799 897, EP 0 785 280, WO 97/31256, WO 97/27317 and WO 98/08083.

As already mentioned above, in these applications, it is envisaged that arrays of the invention can be developed that carry most or even all markers of interest for a specific genotyping collection, such as for a specific species. Other arrays of the invention may contain most or all markers necessary to classify an individual within a genotyping collection, i.e. as belonging to a certain species, subspecies, variety, cultivar, race, strain or line, or to study the inheritance of a genetic trait or property. Also, an array of the invention may contain all markers indicative of the presence, the absence or the state of a genetically determined or genetically influenced disease or disorder, including cancer, oncogenes and oncogenic mutations. Such an array may then be used for diagnostic purposes.

Similarly, it may be possible to provide cDNA-based arrays for any of these purposes.

Some non-limiting examples of species of plants, animals and micro-organisms for which arrays of the invention are particularly envisaged include humans, animals such as mouse, rat, pig, etc., plants such as wheat, barley, maize, tomato, pepper, lettuce, rice, and micro-organisms such as yeast, bacteria and fungi-algi.

In a further aspect, the invention relates to results and/or data obtainable by analysing a nucleic acid or mixture of nucleic acids with an array of the invention. These results or data may for instance be in the form of an image, of a score, of digital or analog data, or in another suitable form, and may optionally be stored on a suitable data carrier, including paper, photographic film, computer disc of files, a database, etc.. This data may be as directly obtained from analysing or scoring the array, or may have been processed further.

The invention will now be further illustrated by means of the following non-limiting Experimental Part, as well as by the enclosed Figures 1-8, which show (representations of) the results of hybridisations using micro-arrays of the invention, obtained by scanning the arrays using the Genetac 1000 scanner (Genomic solutions). The arrays are irradiated with a Xenon-lamp and the signals are detected using a CCD-camera. Filters for Cy-3 and Cy-5 are used. The scantime is about 180 sec.

More specifically, the Figures show:

- Figure 1: Detection of a mixture of 5 rice +2/+3 AFLP markers on an array containing 20 rice +2/+3 AFLP markers.
- Figure 2: Detection of rice AFLP markers amplified in +2/+3 AFLP reactions on an array containing 10 rice +2/+3 AFLP markers.
- Figure 3: Detection of rice AFLP markers amplified in a +2/+2 AFLP reaction on an array containing 20 rice +2/+3 AFLP markers.
- Figure 4: Detection of a rice AFLP markers amplified in rice +2/+2 AFLP reactions on an array containing 20 rice AFLP markers.
- Figure 5: Detection of maize +2/+3 AFLP markers on an array containing 48 maize +3/+3 AFLP markers
- Figure 6: Detection of +2/+3 AFLP markers on an array containing 11 *Arabidopsis* +2/+3 AFLP markers.
- Figure 7: Detection of +2/+2 AFLP markers on an array containing 21 +1/+2

tomato cDNA-AFLP fragments.

- Figure 8: Detection of rice AFLP markers amplified in a +2/+3 AFLP reaction on an array containing 5 rice +2/+3 AFLP markers and 5 sets of oligo's corresponding to the forward and reverse strands of these 5 rice +2/+3 AFLP markers.
- Figure 9: Schematic representation of the related AFLP-Primer Combinations ("ÄPCs") used in Example I. Fig. 9A: 4 related APCs of the Enzyme Combination ("EC") *Eco*RI-*Mse*I; Fig. 9B: APC that can be used to simultaneously amplify the 4 APCs of Fig. 9A; Fig. 9C: 16 related APCs of the EC *Pst*I-*Taq*I; Fig. 9D: APC that can be used to simultaneously amplify the 16 APCs of Fig. 9C.
- Figure 10: Schematic representation of the method used in generating the Array-bound Nucleic Acids Sequences (including the AFLP-amplification);
- Figure 11: Schematic representation of the method for identifying, in/from a plurality of AFLP-fingerprints, polymorphic bands suitable for use in an array of the invention, and for "building up" an array from such polymorphic bands;
- Figure 12: Schematic representation of a method for probing a genomic DNA with an array of the invention, in which the genomic DNA is restricted and amplified using AFLP-methodology prior to contacting with the array.

Experimental Part.

Example I: Generation of AFLP micro-arrays.

The method for generating the AFLP micro-arrays comprises steps (1) - (9). Steps (2) - (5) generally follow conventional AFLP-techniques and protocols, as described in EP-0 534 858. A number of steps of said method, as well as the primers/primer combinations used therein, are schematically shown in Figures 9-11.

1. Selection of the genotyping collection.

A limited number of individuals is selected representing the genetic diversity within a specific group in the best possible way. The selected set of individuals is called the "genotyping collection".

The group chosen will be dependant upon the purpose of the array. For instance, when the array is to be used in breeding, the individuals may be from different varieties, lines, strains, cultivars, or races, belonging to the same species.

The number of different individuals will vary, dependant upon the nature of the genotyping collection, the diversity in said collection, the number of markers desired on the array, etc. Usually, the array will contain the markers from 1 to 10 different individuals.

2. Isolation of genomic DNA and preparation of AFLP template DNA.

Genomic DNA is isolated from the individuals of the genotyping collection and AFLP template DNA is prepared of each individual using a certain Restriction Enzyme Combination, as shown in Figure 10. This is carried out for each individual separately, in a manner known per se, for instance from AFLP, essentially as described in EP-0 534 858.

The Restriction Enzyme Combination will comprise at least one frequent cutter and at least one rare cutter as described above and will depend on the genotyping collection used (i.e. which genus or species.

Suitable Restriction Enzyme Combinations, i.e. providing informative polymorphic bands in the final fingerprint, can be selected on the basis of experience or after some routine experimentation. Some non-limiting examples include *Eco*RI/*Mse*I or *Pst*I/*Taq*I. Usually, when a Restriction Enzyme Combination is known that gives informative results in conventional AFLP-fingerprinting, this combination is also used in preparing the array of the invention.

After the genomic DNA has been isolated and restricted with the Restriction Enzyme Combination, adapters are attached to the resulting fragments to provide AFLP-template DNA. Again, conventional AFLP-adapters can be used, essentially as described in EP-0 534 858.

3. Amplification.

The DNA templates from each of the individuals from the genotyping collection are amplified using selective primers.

Preferably, a large number of AFLP reactions is performed on the genotyping collection using a set of "related AFLP-Primer Combinations", hereinbelow referred to as "related APCs". The APCs mentioned in this Example are also schematically shown in Figures 9A-9D.

Related APCs are combinations of selective AFLP-primers that can be used with the same Restriction Enzyme Combination and that can be amplified simultaneously using a corresponding APC with less selective nucleotides, yielding all AFLP fragments from the related APCs at once. Each of the primers that forms part of an APC is essentially the same as a conventional AFLP-primer in that it contains:

1) a sequence corresponding to (i.e. that can hybridize with) the adapter-sequence of the template, connected at its 3' end to:

2) a (usually small) sequence that corresponds to the part of the template sequence that resulted from the cutting of a restriction site in the original genomic DNA with the restriction enzyme used and the ligation of the restricted fragment to the adapter; and

3) at the 3' end of the primer, a number of so-called selective bases, for which further reference is made to EP-0 534 858.

Each primer of an APC can be represented schematically as:

5'- AAAAAAAAAAAAAA - RRR - NNN -3'

in which N is a nucleotide corresponding to the adapter sequence, R is a nucleotide corresponding to the restriction sequence, N is a selective nucleotide (the number of nucleotides A, R, and N may vary and may be different than shown); or alternatively as

[adapter] - [restr.enzyme] - NNN

in which [adapter] is the adapter sequence, [restr.enzyme] is the restriction sequence, and N is a selective nucleotide.

Each APC will consist of two primers, i.e. one primer for the rare cutter and one primer for the frequent cutter. A set of APCs will comprise a number of such two-primer APCs.

To provide a set of "related APCs", the last selective base at the 3' end of the primer for the frequent cutter, of the primer for the rare cutter, or of the primers for both, may be varied to two or more, and preferably all four of the bases A, T, G and C.

If one of the two primers of an APC is varied to all four bases, this will provide a set of 4 APCs; if both of the primers are varied, a set of 16 APCs will be obtained. Examples of such sets of related AFLP-primer combinations are:

A) a set of 4 related APCs for the Restriction Enzyme Combination *EcoR*I-*Mse*I (Figure 9A):

[Adapter] - [*EcoR*I] - GAC + [Adapter] - [*Mse*I] - TCA

[Adapter] - [*EcoR*I] - GAC + [Adapter] - [*Mse*I] - TCC

[Adapter] - [*EcoR*I] - GAC + [Adapter] - [*Mse*I] - TCG

[Adapter] - [*EcoR*I] - GAC + [Adapter] - [*Mse*I] - TCT

These APCs can be used with the Restriction Enzyme Combination *EcoR*I-*Mse*I and have the selective nucleotides GAC at the *EcoR*I-primer and the selective nucleotides TC at the *Mse*I-primer in common. The AFLP-fragments from these 4 related APCs can be amplified at one with the APC

[Adapter] - [*EcoR*I] - GAC + [Adapter] - [*Mse*I] - TC (Figure 9B)

B) the 16 related APCs for the Restriction Enzyme Combination *Pst*I-*Taq*I (Figure 9C):

[Adapter] - [*Pst*I] - CA + [Adapter] - [*Taq*I] - AGA

[Adapter] - [*Pst*I] - CA + [Adapter] - [*Taq*I] - AGC

[Adapter] - [*Pst*I] - CA + [Adapter] - [*Taq*I] - AGG

[Adapter] - [*Pst*I] - CA + [Adapter] - [*Taq*I] - AGT

[Adapter] - [*Pst*I] - CC + [Adapter] - [*Taq*I] - AGA

[Adapter] - [*Pst*I] - CC + [Adapter] - [*Taq*I] - AGC

[Adapter] - [*Pst*I] - CC + [Adapter] - [*Taq*I] - AGG

[Adapter] - [*Pst*I] - CC + [Adapter] - [*Taq*I] - AGT

[Adapter] - [*Pst*I] - CG + [Adapter] - [*Taq*I] - AGA

[Adapter] - [*Pst*I] - CG + [Adapter] - [*Taq*I] - AGC

[Adapter] - [*Pst*I] - CG + [Adapter] - [*Taq*I] - AGG

[Adapter] - [*Pst*I] - CG + [Adapter] - [*Taq*I] - AGT

[Adapter] - [*Pst*I] - CT + [Adapter] - [*Taq*I] - AGA

[Adapter] - [*Pst*I] - CT + [Adapter] - [*Taq*I] - AGC

[Adapter] - [*Pst*I] - CT + [Adapter] - [*Taq*I] - AGG

[Adapter] - [*Pst*I] - CT + [Adapter] - [*Taq*I] - AGT

These APCs can be used with the Restriction Enzyme Combination *Pst*I-*Taq*I and have the selective nucleotide C at the *Pst*I-primer and the selective nucleotides AG at the *Taq*I-primer in common. The AFLP-fragments from these 4 related APCs can be amplified at once with the APC

[Adapter] - [*Pst*I] - C + [Adapter] - [*Taq*I] – AG (Figure 9D)

Preferably, in an APC, primers with 1, 2, 3 or 4 selective nucleotides are used. More preferably, each APC comprises a combination of two +3-primers, or one +3-primer and one +2-primer, or two +2 primers.

4. Fingerprinting.

After a suitable set of related APCs has been selected, the restricted genomic DNA of an individual from the genotyping collection is amplified using one APC from the set.

This is carried out for all individuals of the genotyping collection, in separate amplifications, carried out essentially as described in EP-0 534 858, which are usually run simultaniously.

The resulting AFLP reactions, one for each individual of the genotyping collection, are then analyzed in parallel on sequencing gels. After electrophoresis, these gels are dried on Whatman 3 MM paper and the AFLP fingerprints are visualized, e.g. by autoradiography or phospho-imaging.

In this way the AFLP fingerprints of the individuals of the genotyping collection are displayed side by side on the fingerprint. This is schematically shown in Figure 11, in which the AFLP-reactions of a genotyping collection of 4 individuals (referred to "ind.1" to "ind.4" – and corresponding to the lanes from left to right in the gels) have been visualised in the four parallel lanes of each of the gels "pk 1" to "pk 4" (in which each gel was generated with a different APC from a set of four related APCs).

5. Identification of polymorphic bands.

The AFLP fingerprints of the individuals of the genotyping collection are inspected for AFLP fragments that reveal DNA polymorphisms; such AFLP fragments are called "AFLP markers". This is again schematically shown in Figure 11, in which each marker has been circled. Bands that are the same for each individual fingerprint are not selected. These bands are then assembled on the array, i.e. as described hereinbelow. This method is also exemplified by the method schematically shown in Figure 12.

6. Isolation of the AFLP-markers.

The AFLP markers are cut out from the gel with the gel piece and the attached Whatman 3 MM paper. This is carried out for each marker separately.

7. Purification, reamplification and cloning.

The AFLP markers thus identified and separated are eluted from their respected gel pieces and separately reamplified using the AFLP primers (i.e. the APC) initially used to generate the AFLP fingerprint from which the AFLP marker is derived. Next, the AFLP markers are cloned into appropriately digested plasmid vectors according to standard procedures.

8. Generating an AFLP fragment library.

The procedure of steps 6 and 7 is repeated for the various APCs of a set of related APCs.

This is again schematically shown in Figure 11, in which each of the gels pk 1 to pk 4 has been generated using one of the APCs from a set of four (and each gel contains, in each parallel lane, the fingerprint of one individual of the genotyping collection obtained with the APC used).

In this way an AFLP fragment library is build up containing AFLP markers identified using the genotyping collection.

EXAMPLE II: Carrier attachment and formation of an array.

The individual AFLP markers of the AFLP fragment library are attached to a carrier; many different AFLP fragments are attached to the same carrier. This is preferably carried out according to a predetermined pattern, in which for instance the markers generated from the genotyping collection with a specific APC are grouped together, i.e. as a column as shown in Figure 11.

Also, the markers generated with each of the APCs from the set of related APCs may be grouped together, to form a set of lines, rows or columns, or a rectangle, as is shown in Figure 11.

In this way an array of AFLP markers is created on the carrier. In case of a high-density array such arrays are called AFLP micro-arrays. Usually, each APC will provide about 10-50 markers, depending upon the genotyping collection and the number of individuals used.

The array thus obtained can then be used to probe the genomic DNA of a further individual for the presence of the AFLP markers attached to the array, as further described in Example III. Usually, this further individual will belong to or be related to the genotyping collection used in generating the array, or at least will be suspected of containing in its genome one or more of the markers present on the array.

EXAMPLE III: Genotyping using AFLP micro-arrays.

This procedure uses the AFLP micro-arrays obtained as described in Example I. Such micro-arrays contain a multitude of AFLP markers derived from a specific genus. (In general AFLP markers will be genus-specific and AFLP markers generated from a different genus will usually not be usable for genotyping of individuals from other genera).

Genotyping of a specific individual can be performed by investigating the presence or absence of each AFLP marker of the AFLP micro-array in the individual tested. This can for instance be achieved by hybridization of a collection of AFLP-fragments from the individual to the AFLP markers attached to the micro-array.

This collection of AFLP fragments is preferably generated from the individual of interest by AFLP amplification of AFLP template DNA of the individual. The

collection of AFLP fragments can be labeled to enable the detection of the AFLP fragments hybridized to their counterparts on the AFLP micro-array.

In general, this procedure comprises the following steps:

1. Isolation of genomic DNA and preparation of AFLP template DNA.

Genomic DNA is isolated from the individual tested and AFLP template DNA is prepared. This is carried out in a manner known per se, for instance essentially as described in EP-0 534 858.

Preferably, the same Restriction Enzyme Combination is used as was used in generating the template DNA for the array. More preferably, a method analogous to the method of step 2 of Example I is used, i.e. following the same or a similar protocol.

However, the adapters used are preferably chosen such that they do not hybridize with the adapter sequences present in the Array-bound Nucleic Acid Sequences (if any). Such hybridization between the adapter sequences could give rise to false-positive signals (i.e. not corresponding to the presence of an AFLP marker in the sample to be tested), in particular if low stringency hybridization conditions are used.

To avoid this, in the preparation of the template DNA to be tested with the array, different adapters to those used in generating the array may be used. Alternatively, and preferably, the adapter sequences present in the AFLP markers isolated from the gel in step 6 of Example I above are either removed or replaced by other adapter sequences prior to attachment of the marker to the array (but usually after reamplification of the isolated markers in step 7 of Example I). This may be achieved during cloning of the AFLP fragments as described in Procedure 7 of Example I.

2. Amplification and labelling.

A single AFLP reaction is performed on the template DNA obtained in step 1, using an APC that corresponds to the Restriction Enzyme Combination, to generate the AFLP fragments specific for the APC selected.

Preferably, said APC is further selected to include all APCs from the set of related APCs used to generate the markers on the AFLP micro-array, or at least a subset thereof. Generally, this means that one or both of the primers of said APC will contain

less selective nucleotides than the primers of the set of related APCs.

Usually, the primers of said APC will contain no selective bases on the positions varied in the primers of the set of "related APCs" used in generating the array, as exemplified in step 3 of Example I above. The remainder of the selective bases in primers of said APC will be the same as in the primers of the set of related APCs, also as exemplified in step 3 of Example I.

In principle, using said APC, all fragments that have been amplified separately with the set of related APCs can be amplified together. Therefore, by using said APC on the template DNA of step 1, a mixture of amplified fragments can be generated that will contain any marker generated with the set of related APCs, if such a marker is/was also present in the genomic DNA to be tested.

For the remainder, the amplification is carried out in a manner known per se, for instance essentially as described in EP-0 534 858, and preferably in a manner analogous to step 3 of Example I, i.e. following the same or a similar protocol.

During or after amplification, the AFLP fragments are labeled by using end-labeled AFLP primers, or by internal labeling. The label may be a fluorescent label, a radio-active label, or other types of labels suitable for detection on micro-arrays.

3. Hybridization with the array.

The labeled AFLP fragments generated with the selected APC are used as a probe in a hybridization to the AFLP fragments on the AFLP micro-array. The collection of labeled AFLP fragments is called the "AFLP target". AFLP markers represented on the AFLP micro-array will hybridize to their labeled counterparts in the AFLP target, provided that these AFLP markers are present in the individual selected. The result is that the AFLP markers on the array that correspond to markers present in the individual tested will hybridize to their labeled counterparts, and give a positive hybridization signal on the array (i.e. show the presence of the label).

AFLP markers on the array that are not present in the individual tested will not find corresponding labelled sequences in the amplified sample, and will therefore not give a positive signal.

4. Scanning, detection and analysis of the array.

After hybridization, the AFLP micro-array is scanned visually or using automated equipment. Each spot harboring an AFLP marker present in the individual will show the presence of the label, spots representing AFLP markers absent in the individual will not be labeled. In this way the presence or absence of each AFLP marker on the AFLP micro-array in the individual tested can be assessed. These results may also be referenced further by comparison to earlier results obtained with the same array, or be stored in a database for future reference.

EXAMPLE IV: Procedure for generating AFLP fragments for use in micro arrays.

This method generally comprises the steps of:

1. Isolation of the fragments from an AFLP gel
2. Reamplification of the isolated fragments using primers that reconstitute the restriction sites
3. Purification and digestion of the reamplified products
4. Cloning of the fragments in pUC vector
5. Validation of the clone fragments by fingerprinting pools of cloned fragments (obtained using colony PCR), and comparing the fingerprints thus obtained with the original fingerprints used in step 1.

1. Isolation of the AFLP fragments from the gel

AFLP reactions are carried out using 10 ng Mse primer and 30 ng primer for the rare cutter (of which 5 ng is kinased with ^{33}P γ-d-ATP) The AFLP reactions are run on standard 4.5% gradient gel. The gel is transferred to Whattman-3MM paper and dried. The dried gel is exposed (>o/n)with a sensitive photographic film. The fragments to be spotted on the micro array are cut out from the gel as a thin slice (about 1 mm) and transferred to 100 µl $TE_{0,1}$

2. Reamplification

*Eco*RI+A/*Mse*I+C fragments are reamplified with the following primers 98L19 and 98L20, that reconstitute the restriction sites.

98L19:AGCGGATAACAATTTCACACAGGATAGACTGCGTACGAATTCA
M13 reverse sequence primer X
E01: GACTGCGTACCAATTCA

98L20:CGCCAGGGTTTTCCCAGTCACGACGATGAGTCCTGATTAAC
M13 forward sequence primer X
M02: GATGAGTCCTGAGTAAC

(98L19 = SEQ ID no.1 ; E01 = SEQ ID no.2 ; 98L20 = SEQ ID no. 3; M02 = SEQ ID no.4)

The PCR reaction mixture is as follows: 5 µl eluate; 150 ng 98L19; 150 ng 98L20; 2 µl 5 mM dNTP's; 5 µl 10 x PCR buffer; 0.2 U Taq polymerase, in a total volume of 50 µl. The PCR profile is as follows: 30 sec. 94°C : 30 sec. 56°C : 1 min. 72°C, for 30 cycles.

*Pst*I+A/*Mse*I+C fragments are reamplified with primers 98/L88 and 98/L20

98L88:AGCGGATAACAATTTCACACAGGATAGACTGCGTACCTGCAGA
M13 reverse sequence primer X
P01 GACTGCGTACATGCAGA
X
Ps01 GACTGCGTACCTGCAGA

(98L88 = SEQ ID no.5 ; P01 = SEQ ID no.6 ; Ps01 = SEQ ID no.7) or optionally with 98L89/98L20.

98L89:AGCGGATAACAATTTCACACAGGATAGACTGCGTACCTGC
M13 reverse sequence primer X
P00: GACTGCGTACATGCAG
(98L89 = SEQ ID no.8 ; P00 = SEQ ID no.9)

3. Purification and digestion of the reamplified fragments

The PCR-reactions are purified using a Qiaquick 96-well PCR centrifugation kit (Qiagen) according to the manufacturers protocol.

The elution step is carried out using 80 µl elution buffer, to a final volume of about 50 µl. The elution volume is collected on a microtiter plate. The purified PCR-products are restricted by adding 5U rare cutter enzyme, 5U MseI to a total volume of 74 µl 1x RL+, and the mixture is incubated for 2 hours at 37°C. After the restriction/digestion, the DNA (on the microtiter plate) is precipitated with isopropanol by adding 7.5 µl 3M NaOAc, 85 µl isopropanol, and the mixture is kept at room temperature for 15 min and then centrifuged for 45 min (3500 rpm). Excess isopropanol is removed and the microtiter plates are again centrifugated (10 sec. at 1000 rpm). The pellet (not visible) is taken up in 15 µl $TE_{0,1}$, and a 3 µl aliquot thereof is checked on the 2% agarose gel.

4. Cloning of the reamplified fragments

The ligation reaction is carried out in the following mixture: 7 µl reamplification product (in PCR base); 8 µl ligation mixture; 100 ng PstI- or *Eco*RI/*Nde*I-restricted, gel-purified pUC18; 3 µl 5X RL^+;1.5 µl 10 mM ATP; 1U T_4 DNA Ligase to a total volume of 8 µl. The mixture is incubated (o/n) at room temperature.

The transformation (in PCR base) is carried out as follows. 7.5 µl ligation reaction is kept on ice, 50 µl frozen competent DH5α cells are added (on ice), and the mixture is incubated for 30 min. (on ice). The mixture is then subjected to a heat shock (42°C) during 90 sec and kept on ice for 2 min, after which 200 µl TY medium is added, and the mixture is allowed to recover (1 hour at 37°C). 200 µl of the mixture is plated on TY+carbeniciline agar plate and incubated (o/n) at 37°C.

5. Reamplification and validation of the cloned fragments

The following reamplification primers are used:

- rare cutter side: 98L58: GGAAACAGCTATGACCATGATTAC (pUC 18 primer, SEQ ID no.10)
- *Nde*I side: 98L55 GATTGTACTGAGAGTGCACCTTAAC (pUC 18 primer, with reconstituted *Mse*I site, only for Mse+C, SEQ ID no.11).

For each clone fragment 3 different clones and inoculated into 10 µl TY, followed by incubation (o/n) at 37°C. *E. coli* cells are transferred to 96-wells plate with 50 µl $TE_{0,1}$ per well, and 5 µl is transferred to PCR base. The PCR base is incubated at 95°C during 5 min, after which 45 µl PCR mixture is added, which comprises: 75 ng primer 98L58; 75 ng primer 98L55; 2 µl 5 mM dNTP's; 5 µl 10X PCR buffer; 0.25 µl Taq polymerase; 0.85 µl 10 mg/ml BSA, to a total volume of 45 µl. The PCR profile is as follows: 25 sec. 94°C; 30 sec. 56°C; 1 min. 72°C; for 30 cycles. 5 µl of the mixture is checked on the gel.

For each APC from which a fragment is obtained, 3 pools are made. Pool A contains fragment 1, 4, 7, 10 ...; Pool B contains fragment 2, 5, 8, 11 ...; Pool C contains fragment 3, 6, 9, 12 .... For each clone 5 µl colony PCR material is pooled, and 5 µl of each pool us used for a template preparation (standard AFLP template).

The template is checked by standard AFLP reaction of 1/10 diluted pool template, the fingerprint of which is compared to the fingerprint from which the original fragments were obtained.

EXAMPLE V: Protocols for detecting AFLP fragments using micro arrays.

Preparing the micro arrays

The micro arrays are prepared using DNA probes that are synthesized via "colony PCR" using pUC18 specific primers. For preparing the arrays, DNA solutions at a concentration of about 0.5 µg/µl are used. Diluted colony PCR material is used for routine synthesis of probe DNA.

1. Amplification of the probes

To synthesize as much DNA as possible in a small a volume as possible, an

adaptation of a conventional PCR protocol is used (increased amounts of primer, dNTP's and $MgCl_2$ are added). The PCR mixture is as follows: 5 µl 1/400 preamp; 6.3 µl primer 1 (50 ng/µl); 6.3 µl primer 2 (50 ng/µl); 8.4 µl dNTP (5 mM); 3.36 µl $MgCl_2$ (25 mM); 10.5 µl PCR buffer (10x); 0.525 µl Taq DNA Polymerase (5 U/µl); H_2O to 105 µl final volume. The PCR profile is as follows: 30 sec. 94°C; 30 sec. 55 °C; 1 min. 72°C; 30 cycles; PE 9600 MODE. The gel reference is 2.5 µl PCR on 2% agarose gel. The following primers are used: Standard: 98L55 + 98L58; with 5'NH_2: 98L59 (NH_2) + 98L58 NH_2; with 5' Cy-3 and internal NH_2: 98L59 (Cy-3, NH_2) + 98L58 (Cy-3, NH_2).

On the basis of 315 ng of each primer, theoretically 8 µg product can be formed in the reaction in a 105 µl PCR (assuming an average fragment length of 250 bp and that all primer is used). For a PCR efficiency of 80%, 6.4 µg product will be synthesized. Thus, for 50 µl product at a concentration of about 0.5 µg/µl, 3 PCR reactions of 105 µl are necessary.

2. Precipitation of PCR reactions:

The precipitation of the PCR reactions is carried out as follows. To 3x 105 µl PCR reaction + 31.5 µl (1/10 volume) 3M NaAc is added 346 µl (1 volume) 2-propanol, and the mixture is kept for 30 min at -20°C. The mixture is then centrifuged (30 min, 13000 rpm, 4°C) and the pellet is washed with 100 µl 70% EtOH. The mixture is then again centrifuged (10 min, 13000 rpm, RT) and the pellet is dried to air, dissolved in 25 µl H_2O and 25 µl DMSO is added. As a gel reference 0.5 µl on 2% agarose gel is used.

3. Preparation of the arrays

To prepare ("print") the arrays a GMS 417 arrayer (Genetic Microsystems) is used. Such an arrayer can be configured according to a variety of variables. In preliminary tests, a number of standard settings are used, making the lay-out of all arrays comparable. A good means for localizing the spots is the use of labelled primers -in particular Cy-3 or Cy-5-primers- for making the probes. This makes the position of the printed sequences ("spots") clearly visible on the scans of the arrays

and serves as a control to monitor deposition and binding to the array.The slides used were EMS Poly-L-lysine slides (Electron Microscopy Scienses, Washington).

Printing of the slides is carried out as follows:

a) Position of the slides:

- Piece of "matted glass" on the slides on the left side (against the clamp).
- Press slides well together.

b) Position of the microtiter plates:

- A1 coordinate of the plates left side front in the plate holders.

c) Settings arrayer:

1x spotting X = 3 Y = 15, duplo X = 7.4 Y = 15

5x spotting X = 3 Y = 19, duplo X = 7.4 Y = 19

spot spacing: 300 μm - 350 μm.

To limit the evaporation of the probe DNA's, the microtiter plates can be kept above a bath of warm water.

4. Processing of the arrays

During the processing of the arrays, DNA is adhered to the glass carrier and denaturated, depending upon the type of slide and the coating. The processing of EMS Poly-L-lysine slides is carried out as described by P. Brown (http://cmgm.stanford.edu/pbrown/protocols/3_post_process.html):

The slide is rehydrated on top of a hot water bath for 1 minute, so that the slide becomes fogged, and snap dried on a heated cooking plate (about 3 sec.). The slide is then rehydrated for 10 sec, UV cross-linked at 65 mJ (Amersham UV crosslinker at 650 x 100 μJ), and incubated for 15-20 min in blocking solution (in a glass tray), with gentle agitation. The blocking solution comprises 325 ml 1-methyl-2-pyrrolidone (100 ml); 6 g succinic anhydride (1.8 g) and 15 ml sodium borate (pH 8.0) (4.6 ml). The slides are washed for 2 min. in H_2O (95°C); washed for 1 min. in 96% ethanol; and dried for 5 min. by centrifugation in a tabletop centrifuge at 1000 rpm.

5. Labeling of the target reactions

For the labeling of the target DNA several methods can be used. Hereinbelow, a labeling method will be used in which Cy-3 or Cy-5 labelled dCTP molecules are enzymatically incorporated into the target DNA using Klenow DNA polymerase.

The PCR reaction was as follows: PCR reaction: 5 µl 1/400 preamp or 5 µl 1: 10 AFLP template; 6.3 µl primer 1 (50 ng/µl); 6.3 µl primer 2 (50 ng/µl); 3.36 µl $MgCl_2$ (25 mM); 8.4 µl dNTP (5 mM); 10.5 µl Pcr buffer (10x); 0.525 µl Taq DNA Polymerase (5 M/µl); and H_2O until 105 µl final volume. The PCR profile is dependent upon the AFLP extension reaction. If only one selective nucleotide is used, a stable profile is used, e.g.. 30 sec. 55°C; 1 min. 72°C; for 30 cycles, PE 9600 MODE. With more than one selective nucleotide, a standard AFLP profile (with touch down) is used, e.g 30 sec. 94°C; 30 sec. 65°C 1 cycle; 1 min. 72°C; followed by lowering of the annealings temp. with 0.7°C during 12 cycles (in total 13 cycles touch down); 30 sec. 95°C; 30 sec. 56°C - 23 cycles; 1 min. 72°C

The target reaction is precipitated as follows. 10 µl (1/10 volume) 3 M NaAc is added to 100 µl target PCR reaction. 110 µl (1 volume) 2-propanol is added, and the mixture is kept for 30 min at -20°C. The mixture is then centrifuged (30 min at 13000 rpm and 4°C) and the pellet is washed with 100 µl 70% EtOH, followed by centrifugation (10 min at 13000 rpm and RT). The pellet is then dried to ambient air and taken up in 10 µl H_2O.

The preparation of labeled target DNA using Klenow DNA Polymerase was carried out as follows. To 5 µl target DNA (about 3-6 µg AFLP reaction) and 2.5 µl AFLP primer (1 µg/µl) is added H_2O to a total volume of 20 µl, and the mixture is kept for 5 min. at 95°C and then cooled to room temperature. Then are added: 5 µl dCTP with Cy-3 or Cy-5 label (0.5 mM); 1 µl 5 mM of each of dATP, dGTP, dTTP; 5 µl 10x T4 buffer; 2.5 µl Klenow DNA Pol (8u/µl) and 16.5 µl H_2O, and the mixture is incubated for 2 hours at 37°C

The labeled target reactions are purified using a Qiaquick column, according to the manufacturer's instructions. The elution is in 50 µl elution buffer.

The pellet, which must be clearly stained, is dissolved in in 18 µl H_2O for Klenow target or in 15 µl H_2O for Mirus target.

8. Hybridisation

The denaturation of labeled target is carried out by adding 1.5 µl denaturation buffer D1 (3M NaOH), after which the mixture is kept at room temperature for 5 min, and then place on iced, upon which 1.5 µl neutralisation buffer N1 (1M Tris pH 7.3, 3M HCl) is added.

Subsequently, 18 µl 2x hybridization buffer (preheated), comprising 4x SSC, 5x Denhardt, 0.5% SDS, is added at 60°C, after which the hybridization is started by, with a pipet, adding 30 µl target solution to the slide, next to the array.
A the cover glass (24x50 mm) is placed in position (without air bubbles), and the slides are incubated (o/n) at 45°C in a single incubation chamber (in which case 2 drops 10 µl 3x SSC are added) or in a large incubatotion tank containing water.

The hybridizations are washed by rinsing with 4x SSC 0.1% SDS (45°C); incubating for about 5 min in 2x SSC 0.1% SDS (45°C); incubating for 5 min 1x SSC 0.1% SDS (RT); incubating for 5 min in 0.5x SSC 0.1% SDS (RT); incubate for 2 min in 0.5x SSC (RT); followed by centrifugation for 10 min (500 rpm) in a tabletop centrifuge.

9. Scanning

The arrays are scanned using the Genetac 1000 scanner (Genomic solutions). The arrays are irradiated with a Xenon-lamp and the signals are detected using a CCD-camera. Filters for Cy-3 and Cy-5 are used. The scantime is about 180 sec.

EXAMPLE VI: Detection of AFLP markers on microarrays.

EXAMPLE VI-1: Detection of a mixture of 5 rice +2/+3 AFLP markers on an array containing 20 rice +2/+3 AFLP markers.

An array of 20 rice +2/+3 AFLP markers (probes) was prepared from cloned

AFLP markers generated using restriction enzymes *Eco*RI and *Mse*I and parental lines IR20 and 6383. The AFLP marker name, AFLP primer combination (PC) used, estimated mobility (size in basepairs) and the parental origin of these 20 AFLP markers are:

	PC	Size (b.p.)	Parent Line
1.	E11/M47	145	IR20
2.	E11/M50	342	IR20
3.	E11/M50	173	IR20
4.	E11/M50	143	IR20
5.	E11/M50	101	IR20
6.	E11/M49	583	IR20
7.	E11/M49	243	IR20
8.	E11/M49	210	IR20
9.	E11/M49	200	IR20
10.	E11/M49	196	IR20
11.	E11/M47	160	6383
12.	E11/M50	214	6383
13.	E11/M49	342	6383
14.	E11/M49	299	6383
15.	E11/M49	273	6383
16	E11/M49	247	6383
17.	E11/M49	194	6383
18.	E11/M49	159	6383
19.	E11/M49	149	6383
20.	E11/M49	146	6383

The of sequences the +2/+3 AFLP primers used to generate these 20 AFLP markers are:

EcoRI	E11:	5'-GACTGCGTACCAATTCAA-3'	(SEQ ID no.12)
MseI	M47:	5'-GATGAGTCCTGAGTAACAA-3'	(SEQ ID no.13)
	M49:	5'-GATGAGTCCTGAGTAACAG-3'	(SEQ ID no.14)
	M50:	5'-GATGAGTCCTGAGTAACAT-3'	(SEQ ID no.15)

The AFLP reactions used to isolate the 20 AFLP +2/+3 makers were generated and resolved on sequence gels using the standard procedure (Vos et al., Nucleic Acids Research 23; 4407-4414, 1995 and EP 0 534 858). The AFLP markers were excised from a sequencing gel after transfer to Whatmann paper, followed by drying and exposure to X-ray film to visualize the fingerprint pattern and reamplified using primers :
5'-AGCGGATAACAATTTCACACAGGATAGACTGCGTACGAATTCA-3' (SEQ ID no.16) and
5'-CGCCAGGGTTTTCCCAGTCACGACGATGAGTCCTGATTAAC-3' (SEQ ID no.17) as described in the protocol.

After cutting with *Eco*RI and *Mse*I and purification using Qiagen PCR purification kits (Qiagen) the restricted AFLP marker fragments were cloned in plasmid vector digested with *Eco*RI and *Nde*I. After transformation to *E.coli*, recombinant clones were validated for the correct size insert by AFLP fingerprint analysis of pooled amplified clone inserts. The inserts of clones with validated inserts were sequenced using a standard dye terminator cycle sequencing kit (ABI) according to standard protocols supplied by the manufacturer.

Insert DNAs of individual validated clones were amplified from bacterial stocks by PCR using either unlabelled vector primers or Cy3-labelled vector primers as described (see protocol enclosed) and the PCR reactions were precipated using n-propanol and sodiumbicarbonate according to standard procedures. DNAs were resuspended in 50% DMSO to a final concentation of around 500 nanograms per microliter.

Microarrays were prepared by depositing in duplo around 250 picoliters (1

time spotting) of either the unlabelled or the Cy3-labelled DNA solutions onto an EMS poly-L-lysine slide, (Electron Microscopy Scienses, Washington) using a GMS417 microarrayer (Genetic Microsystems, Woburn, MA). The slide was processed according to standard procedures (see protocol enclosed) and hybridized overnight at 45 °C temparature with a mixture of 5 AFLP markers (target) named 8, 10, 15, 17, and 19 as indicated above, after labelling with Cy5 dye (Amersham Pharmacia Biotec) by Klenow enzyme according to standard procedures (see protocol enclosed). After washing according to the protccol, the slide was scanned at the Cy3 channel for 300 seconds (Figure 1B) and at the Cy-5 channel in the automatic exposure mode ("auto"; Figure 1C) using a Genetac1000 microarray slide scanner (Genomic Solutions, Ann Arbor, MI).

The superimposed image of both channels is shown in Figure 1A with annotation to facilitate interpretation of the spotting pattern. Figure 1 shows:

1) uniform deposition of all AFLP probes (red, green or yellow signal of all probes on false-color image after hybridization).
2) specific hybridization to the 5 expected AFLP probes 8, 10, 15, 17 and 19 (Cy5 channel, green on false-color image; in combination with Cy3-labelled fragments yellow on false-color image).
3) no hybridization to the remaining 15 AFLP probes 1, 2, 3, 4, 5, 6, 7, 9, 11, 12, 13, 14, 16, 18 and 20).

EXAMPLE VI-2: Detection of rice AFLP markers amplified in +2/+3 AFLP reactions on an array containing 10 rice +2/+3 AFLP markers.

An array containing 10 rice AFLP markers (probes) was prepared exactly as described in Example VI-1. The array was processed as described and hybridized with a target consisting of a mixture of a Cy5-labelled AFLP +2/+3 reaction (target) derived from parental line 6383 and IR20, prepared with AFLP primers E11 and M49. With this primer combination, parental line IR20 is known to contain AFLP markers 6, 8 and 10 and line 6383 is known to contain AFLP markers 14, 16, 18 and 20 as described in Example VI-1. The array was washed according to the conditions as described in Example VI-1 or protocols refered to in Example VI-1.

Following washing, images were taken at the Cy3 (Figure 2B) and Cy5 (Figure 2C) channels as described in Example VI-1 and the two images were superimposed electronically (Figure 2A). Figure 2 is annotated to facilitate interpretation of the spotting pattern and shows:

1) uniform deposition of all AFLP probes (red, green or yellow signal of all probes on false-color image after hybridization).
2) specific hybridization of the IR20 and 6383 AFLP markers 6, 8, 10, 14, 16, 18 and 20; Cy5, green signal on false-color image hybridized to unlabelled probes and green/yellow signal on false-color image hybridized to Cy3 labelled probes).
3) no hybridization of to the remaining AFLP markers 2, 4 and 12; Cy3, red signal on false-color image).

EXAMPLE VI-3. Detection of rice AFLP markers amplified in a +2/+2 AFLP reaction on an array containing 20 rice +2/+3 AFLP markers.

An array containing 20 rice AFLP markers (probes) was prepared exactly as described in Example VI-1. The array was processed as described and hybridized using a Cy5-labelled AFLP +2/+2 reaction (target) derived from parental line 6383, prepared with AFLP primers E11 and M15: 5'-GATGAGTCCTGAGTAACA-3' (SEQ ID no.18). This parental line is known to contain AFLP markers with names 11, 12, 13, 14, 15, 16, 17, 18, 19 and 20 as described in Example VI-1. The array was washed according to the conditions as described in Example VI-1 or protocols refered to in Example VI-1.

Following washing, images were taken at the Cy3 (Figure 3B) and Cy5 (Figure 3C) channels as described in EXAMPLE VI-1 and the two images were superimposed electronically (Figure 3A). The superimposed image is shown in Figure 3A with annotation to facilitate interpretation of the spotting pattern. Figure 3 shows:

1) uniform deposition of all AFLP probes (red, green or yellow on false-color image).
2) specific hybridization to the AFLP markers in positions 11, 12, 13, 14, 15, 16, 17, 18, 19 and 20 (Cy5; green signal on false-color image; in combination with Cy3

labelled AFLP probes yellow signal on false-color image)

3) (cross)-hybridization to the IR20 derived AFLP probes in positions 2 and 3 (Cy5; green signal on false color image), probably due to co-amplification of AFLP fragments with sequence similarity to IR20 markers in positions A2 and A3 with AFLP primer combinations E11/M15 from 6383 AFLP template.
4) no hybridization to remaining eight IR20-derived AFLP probes 1, 4, 5, 6, 7, 8, 9 and 10.

EXAMPLE VI-4. Detection of a rice AFLP markers amplified in rice +2/+2 AFLP reactions on an array containing 20 rice AFLP markers.

An array containing 20 rice AFLP markers (probes) was prepared exactly as described in Example VI-1. The array was processed as described and hybridized with a target consisting of a mixture of a Cy3-labelled AFLP +2/+2 reaction (target) derived from parental line IR20, prepared with AFLP primers E11 and M15, and a Cy5-labelled AFLP +2/+2 reaction derived from parental line 6383, also prepared with primer combination E11 and M15 (for primer sequences see Example VI-2). The parental line IR20 is known to contain AFLP markers 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 and 6383 is known to contain AFLP markers 11, 12, 13, 14, 15, 16, 17, 18, 19 and 20 as described in Example VI-1. The array was washed according to the conditions as described in Example VI-1 or protocols referred to in Example VI-1.

Following washing, images were taken at the Cy3 (Figure 4B) and Cy5 (Figure 4C) channels both at 180 seconds exposure time and the two images were superimposed electronically (Figure 4A). The superimposed image of both channels is shown in Figure 4A with annotation to facilitate interpretation of the spotting pattern. Figure 4 shows:

1) uniform deposition of all AFLP probes (red, green or yellow on false-color image after hybridization).
2) specific hybridization of IR20 AFLP markers 1, 4, 6, 7, 9 and 10; Cy3, red on false-color image).
3) specific hybridization of 6383 AFLP markers 11, 14, 15, 17 and 20; Cy5, green on false-color image).

4) Hybridization of both IR20 and 6383 AFLP markers 2, 3, 12, 13 and 18; yellow on false-color image).

5) No hybridization to probes 5, 8, 16 and 19.

EXAMPLE VI-5. Detection of maize +2/+3 AFLP markers on an array containing 48 maize +3/+3 AFLP markers

An array of 48 maize +2/+3 AFLP markers (probes) was prepared from cloned AFLP markers generated using restriction enzymes *Eco*RI and *Mse*I and parental lines B73, Mo17, F2, Co255, DK105 and A7. The AFLP marker name, AFLP primer combination (PC) used, estimated mobility (size in basepairs) and the parental origin of these 48 AFLP markers are:

AFLP Marker Name	PC	Size (bp)	Parent Line
A1	E33/M50	596	Mo17
A2	E33/M50	588	Mo17,Co255
A3	E33/M50	580	B73,A7
A4	E33/M50	566	B73,Mo17,Co255,A7
A5	E33/M50	526	B73,Mo17,Co255
A6	E33/M50	503	F2,DK105
A7	E33/M50	459	DK105
A8	E33/M50	453	B73,DK105
A9	E33/M50	447	B73,Mo17,Co255,DK105,A7
A10	E33/M50	434	Mo17,F2,Co255,
A11	E33/M50	424	F2,Co255,A7
A12	E33/M50	416	B73,Mo17,F2,Co255,DK105,A7
C1	E33/M50	308	Mo17,F2,Co255
C2	E33/M50	304	B73,Mo17,F2,Co255,DK105,A7
C3	E33/M50	292	B73,Mo17,F2,DK105,A7
C4	E33/M50	290	B73,Mo17,F2,Co255,DK105,A7

C5	E33/M50	280	B73,Mo17,A7
C6	E33/M50	274	Mo17,F2,Co255,DK105
C7	E33/M50	269	DK105
C8	E33/M50	264	B73,Mo17,F2,DK105
C9	E33/M50	262	Mo17,Co255
C10	E33/M50	258	B73,A7
C11	E33/M50	255	F2
C12	E33/M50	252	B73,Mo17,Co255,DK105,A7
E1	E33/M50	205	Mo17
E2	E33/M50	204	F2
E3	E33/M50	202	B73,Mo17,A7
E4	E33/M50	201	F2
E5	E33/M50	196	B73,Co255
E6	E33/M50	181	B73,A7
E7	E33/M50	179	Mo17
E8	E33/M50	171	Dk105
E9	E33/M50	169	B73,A7
E10	E33/M50	168	F2
E11	E33/M50	167	B73,F2,A7
E12	E33/M50	161	F2
G1	E33/M50	131	Mo17
G2	E33/M50	128	F2,DK105,A7
G3	E33/M50	127	B73,Mo17,F2,Co255
G4	E33/M50	124	B73,Mo17,Co255,DK105
G5	E33/M50	121	B73
G6	E33/M50	113	Mo17,A7
G7	E33/M50	113	B73,DK105,A7
G8	E33/M50	111	A7
G9	E33/M50	109	F2,Co255
G10	E33/M50	109	Mo17,F2,Co255

G11	E33/M50	106	B73,Mo17,F2,DK105,A7
G12	E33/M50	103	B73,F2,Co255,DK105,A7

The sequences the +2/+3 AFLP primers used to generate these 48 AFLP markers are:

E33: *EcoRI*: 5' GACTGCGTACCAATTCAAG-3' (SEQ ID no.19)

M50: *MseI*: 5'GATGAGTCCTGAGTAACAT-3' (SEQ ID no.20)

The AFLP reactions used to isolate the 48 AFLP +3/+3 makers were generated, excised, reamplified, purified, cloned and validated as described in the protocol of Example VI-1. The inserts of clones with validated inserts were sequenced using a standard dye terminator cycle sequencing kit (ABI) according to standard protocols supplied by the manufacturer.

Insert DNAs of individual validated clones were amplified from bacterial stocks by PCR using either unlabelled vector primers or Cy3-labelled vector primers as described in Example VI-1.

PCR reactions were precipitated and dissolved as described in the protocol of Example VI-1. Microarrays were prepared by depositing in duplo around 250 picoliters (1 time spotting) or 1250 picoliters (5 times spotting) of either the unlabelled or the Cy3-labelled DNA solutions, processed and hybridized according to the protocol of Example VI-1. The target was a mixture of complete +2/+3 E33/M50 AFLP reactions of the parental lines B73 and F2, after labeling the B73 DNA with Cy5 dye (Amersham Pharmacia Biotec), and the F2 DNA with Cy3, by Klenow enzyme according to standard procedures (see protocol enclosed). After washing according to the protocol, the slide was scanned at the Cy5 and Cy3 channels for 180 seconds each using a Genetac1000 microarray slide scanner (Genomic Solutions, Ann Arbor, MI).

The superimposed image of both channels is shown in Figure 5 with annotation to facilitate interpretation of the spotting pattern. Figure 5 shows:

1) uniform deposition of all AFLP probes (red, green or yellow signal of all probes on false-color image after hybridization).

2) specific hybridization of B73 target to expected AFLP probes A3, C5, E9, E11, G7, C10, and E6 (Cy5 channel, green on false-color image);
3) Specific hybridisation of F2 target to expected AFLP probes A6, A8, A11, G7 and G10 (Cy3 channel, red on false-color image);
4) Specific co-hybridization of B73 and F2 targets to expected probes A12, C3, C4, C8 and G12 (Cy5 and Cy3 channels, yellow on false-color image);
5) Strong non-specific cross-hybridisation to probes A9, C7, G5, A8, C2, C6, C12, E8 and G8;
6) Weak non-specific cross-hybridisation to probes A1, E1, G1, G12, A2, A4 and A10;
7) No hybridisation to probes C1, C11, G3, E2, E4, E10, E12, G2 and G4;
8) Specific lack of hybridization to the AFLP probes A1, A9, E1 and G6.

EXAMPLE IV-6. Detection of +2/+3 AFLP markers on an array containing 11 arabidopsis +2/+3 AFLP markers.

Arrays of 11 Arabidopsis +2/+3 AFLP markers (probes) were prepared from cloned AFLP markers generated using restriction enzymes *Eco*RI and *Mse*I and parental lines Columbia and Landsberg erecta. The AFLP marker name, AFLP primer combination (PC) used, estimated mobility (size in basepairs) and the parental origin of these 11 AFLP markers are:

AFLP marker name	PC	Size (basepairs)	Parent Line
1. A3	E11/M62	560	Columbia.
2. A5	E11/M62	512	Columbia.
3. A7	E11/M62	426	Landsberg er.
4. A9	E11/M62	357	Landsberg er.
5. A11	E11/M62	306	Landsberg er.
6. C1	E11/M62	274	Columbia.
7. C3	E11/M62	271	Columbia.
8. C5	E11/M62	207	Landsberg er.

9. C7	E11/M62	171	Columbia.
10. C9	E11/M62	163	Columbia.
11. C11	E11/M62	153	Columbia.

The sequences of the +2/+3 AFLP primers used to generate these 11 AFLP markers are:

E11: *EcoRI*: 5'-GACTGCGTACCAATTCAA-3' (SEQ ID no.21)

M62: *MseI*: 5'-GATGAGTCCTGAGTAACTT-3' (SEQ ID no.22)

The method used to generate the 11 AFLP +2/+3 markers and the preparation and processing of the arrays containing these 11 Arabidopsis AFLP markers is as described in Example VI-1 or protocols refered to in Example VI-1.

The arrays were hybridized with targets consisting of a Cy5-labelled AFLP +2/+3 reaction derived Colombia or Landsberg erecta, which were prepared as described in Example I. The AFLP used to generated the labelled target were E11: 5'-GACTGCGTACCAATTCAA-3' (SEQ ID no.23)
and M62: 5'-GATGAGTCCTGAGTAACTT-3' (SEQ ID no.24). With this primer combination, the parental line Columbia is known to contain the AFLP markers A3, A5, C1, C3, C7, C9 and C11 and parental line *Landsberg erecta* is known to contain the AFLP markers A7, A9, A11 and C5.

The array was washed according to the conditions discribed in Example VI-1 or protocols refered to in Example VI-1. Folowing washing, images of the array were taken at the Cy3 and Cy5 channels with a 180 second exposure time for both channels and the images were superimposed, as described in Example VI-1 (Figure 6). Figure 6A shows:

1) Specific hybridization of the Columbia AFLP markers A3, A5, C1, C3, C7, C9 and C11 (green signals on false-color image).
2) An anonymous Cy3-labeled AFLP fragment at position A1 which marks the start position of the array (red signal on false-color image)

Figure 6B shows:

1) Specific hybridization of the *Landsberg erecta* AFLP markers A7, A9, A11 and C5, (green signal on false-color image).

2) An anonymous Cy3-labeled AFLP fragment at position A1 which marks the start position of the array (red signal on false-color image).

EXAMPLE VI-7. Detection of +2/+2 AFLP markers on an array containing 21 +1/+2 tomato cDNA-AFLP fragments.

Arrays of 21 tomato +1/+2 cDNA fragments (probes) were prepared from cloned cDNA-AFLP fragments using restriction enzymes *EcoRI* en *MseI* and tomato line 52201.

cDNA-AFLP reactions were carried out as described (Vos et al. Nucleic Acids Research 23: 4407-4414 and European Patent Application EP 0534858).

Line	AFLP marker name	PC	Size (basepairs)	Parent
1.	B1	E01/M16	357	52201
2.	B3	E01/M16	346	52201
3.	B5	E01/M16	336	52201
4.	B7	E01/M16	301	52201
5.	B9	E01/M16	284	52201
6.	B11	E01/M16	267	52201
7.	D1	E01/M16	175	52201
8.	D3	E01/M16	159	52201
9.	D5	E01/M16	136	52201
10.	D7	E01/M16	128	52201
11.	D9	E01/M16	122	52201
12.	D11	E01/M16	110	52201
13.	F3	E01/M17	310	52201
14.	F5	E01/M17	264	52201
15.	F7	E01/M17	259	52201
16.	F9	E01/M17	238	52201
17.	F11	E01/M17	208	52201
18.	H3	E01/M17	139	52201

19.	H5	E01/M17	131	52201
20.	H9	E01/M17	114	52201
21.	H11	E01/M17	103	52201

The sequences of the +1/+2 AFLP primers used to generate these cDNA-AFLP fragments are:

*Eco*RI: E01: 5'-GACTGCGTACCAATTCA-3' (SEQ ID no. 25)

*Mse*I: M16: 5'-GATGAGTCCTGAGTAACC-3' (SEQ ID no.26)

*Mse*I: M17: 5'-GATGAGTCCTGAGTAACG-3' (SEQ ID no.27)

The cDNA-AFLP reactions used to isolate the 21 +1/+2 fragments were generated and resolved on sequence gels using the standard procedure. Arrays were prepared according to the procedures described in EXAMPLE VI-1. cDNA-AFLP fragments were spotted in duplo as described in Example VI-1.

The slides were processed according to standard procedures (see protocol enclosed) and hybridised overnight at 45°C temperature with Cy3-labeled +2/+3 AFLP reactions (targets) of the following six tomato lines:

1. *Lycopersicon Esculentum* (L. esc.) accession Moneyberg
2. *L. Peruvianum* accession LA1708
3. *L. Hirsutum* G1209
4. *L. Chmielevski* LA1848
5. *L. Pimpinellifolium* LA722
6. *L .Pennelli* LA716

The sequences of the AFLP primers involved are E12 (5'-GACTGCGTACCAATTCAC-3', SEQ ID no. 28) and M16 (sequence given above, SEQ ID no.26). Labelling with Cy3 dye (Amersham Pharmacia Biotec) by Klenow enzyme was carried out according to standard procedures as described in Example VI-1. After washing according to the protocol, the slides were scanned at the Cy3 channel in the automatic exposure mode using a Genetac1000 microarray slide scanner. The images of all six hybridisations are shown in Figure 7 (A-F) with

annotation to facilitate interpretation of the spotting pattern. Figure 7 shows:

1) Hybridisation of all lines with cDNA-AFLP probes B5 and B11.
2) Hybridisation of *L.* esc. Moneyberg, *L. Hirsutum*, *L. Pimpinellifolium*, *L. Pennelli* with cDNA-AFLP probe D7 (circled).
3) No hybridisation of *L. Peruvianum*, *L. Chmielevski* with cDNA-AFLP probe D7.
4) Signal of Cy3-labelled anonymous cDNA-AFLP fragments deposited at positions B1 and H11 to serve as a marker for the position of the array on the slide.

EXAMPLE VI-8: Detection of rice AFLP markers amplified in a +2/+3 AFLP reaction on an array containing 5 rice +2/+3 AFLP markers and 5 sets of oligo's corresponding to these 5 rice +2/+3 AFLP markers.

An array containing 5 rice AFLP markers (probes) labeled with Cy3 and 5 sets of oligo's corresponding to these AFLP markers was prepared as described in Example VI-1 using 5 of the AFLP markers as described in Example VI-1. The oligo sets, consisting of 2 complementary oligo's, corresponding to these AFLP markers are stated below.

AFLP markernumber	Forward oligo name	Reverse oligo name
2	99f03	99f04
4	99f07	99f08
6	99f11	99f12
8	99f69	99f70
10	99f19	99f20

Oligoname	Oligo sequence	
99f03	5'-GTCCTCATCAAGTAATAGTCAG-3'	(SEQ ID no.29)
99f04	5'-CTGACTATTACTTGATGAGGAC-3'	(SEQ ID no.30)
99f07	5'-CTTGATCAGGAAGACTTTACTC-3'	(SEQ ID no.31)
99f08	5'-GAGTAAAGTCTTCCTGATCAAG-3'	(SEQ ID no.32)
99f11	5'-CTTCATTTATCCTCGATACATG-3'	(SEQ ID no.33)
99f12	5'-CATGTATCGAGGATAAATGAAG-3'	(SEQ ID no.34)

99f69	5'-GGCAATGCAAGTAGATACTTC-3'	(SEQ ID no.35)
99f70	5'-GAAGTATCTACTTGCATTGCC-3'	(SEQ ID no.36)
99f19	5'-CAGTGTGCTAGTTGATTCCAG-3'	(SEQ ID no.37)
99f20	5'-CTGGAATCAACTAGCACACTG-3'	(SEQ ID no.38)

The array was processed as described and hybridized with a target, consisting of a mixture of equal volumes of Cy5-labelled AFLP +2/+3 reactions (target) derived from the parental lines IR20 and 6383, prepared with AFLP primers E11 and M49. Thus in the labeled target one of the strands of the AFLP +2/+3 reaction fragments is labeled with Cy5. The mixture of parental lines IR20 and 6383 is known to contain AFLP markers 6, 8 and 10 as described in Example VI-1. The array was washed according to the conditions as described in Example VI-1 or protocols referred to in Example VI-1.

Following washing, images were taken at the Cy3 (Figure 8B) and Cy5 (Figure 8C) channels both at 180 seconds exposure time and the two images were superimposed electronically (Figure 8A). The superimposed image of both channels is shown in Figure 8A with annotation to facilitate interpretation of the spotting pattern. Figure 8 shows:

1) uniform deposition of all AFLP probes (red, green or yellow on false-color image after hybridization).
2) specific hybridization to AFLP markers 6, 8 and 10; Cy5, green on false-color image).
3) specific hybridization to reverse sequence oligo's corresponding to the unlabeled strand of the AFLP markers 6, 8 and 10; Cy5, green on false-color image).
4) No hybridisation to the forward sequence oligo's corresponding to the labeled strand of the AFLP markers 6, 8 and 10.
5) No hybridization to AFLP markers or oligo's corresponding to AFLP markers 2 and 4.